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Certificate
PATENT OFFICE

REPUBLIEK VAN SUID-AFRIKA

DEPARTMENT OF TRADE
AND INDUSTRY

Hiermee word gesertifiseer dat
This is to certify that

the documents annexed hereto are true copies of.

Application forms P.1 and P.3, and the provisional specification of South African Patent Application No 1998/9427 as originally filed in the Republic of South Africa on 15 October 1998 in the name of PROTEIN RESEARCH TRUST for an invention entitled "TRANSFORMATION PROCESS".

Geteken te PRETORIA in die Republiek van Suid-Afrika, hierdie 28th dag van May 2003
Signed at PRETORIA in the Republic of South Africa this 28th day of May 2003

Registrar of Patents
Registrar of Patents

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978
APPLICATION FOR A PATENT AND
ACKNOWLEDGEMENT OF RECEIPT
(Section 30(1) Regulation 22)

REPUBLIC OF SOUTH AFRICA
FORM P.1 **REVENUE**
(to be lodged in duplicate)

15.10.98 **R 060.00**

INKOMSTE
REPUBLIEK VAN SUID AFRIKA

THE GRANT OF A PATENT IS HEREBY REQUESTED BY THE UNDERMENTIONED APPLICANT ON THE BASIS OF THE PRESENT APPLICATION FILED IN DUPLICATE

A&A REF HASR V13065

21	01	PATENT APPLICATION NO	989427

71	FULL NAME(S) OF APPLICANT(S)

PROTEIN RESEARCH TRUST

ADDRESS(ES) OF APPLICANT(S)

P O BOX 671, PARKLANDS, 2121, REPUBLIC OF SOUTH AFRICA

54	TITLE OF INVENTION

TRANSFORMATION PROCESS

Only the items marked with an "X" in the blocks below are applicable.

[ ] THE APPLICANT CLAIMS PRIORITY AS SET OUT ON THE ACCOMPANYING FORM P.2. The earliest priority claimed is

Country: -------- No: -------- Date: --------

[ ] THE APPLICATION IS FOR A PATENT OF ADDITION TO PATENT APPLICATION NO 21 01

[ ] THIS APPLICATION IS A FRESH APPLICATION IN TERMS OF SECTION 37 AND BASED ON APPLICATION NO 21 01

THIS APPLICATION IS ACCOMPANIED BY:

[X] A single copy of a provisional specification of 9 pages
[ ] Drawings of sheets
[ ] Publication particulars and abstract (Form P.8 in duplicate) (for complete only)
[ ] A copy of Figure of the drawings (if any) for the abstract (for complete only)
[ ] An assignment of invention
[ ] Certified priority document(s). (State quantity)
[ ] Translation of the priority document(s)
[ ] An assignment of priority rights
[ ] A copy of Form P.2 and the specification of RSA Patent Application No 21 01
[X] Form P.2 in duplicate
[X] A declaration and power of attorney on Form P.3
[ ] Request for ante-dating on Form P.4
[ ] Request for classification on Form P.9
[ ] Request for delay of acceptance on Form P.4
[ ] Extra copy of informal drawings (for complete only)

74	ADDRESS FOR SERVICE: Adams & Adams, Pretoria

Dated this **15TH** day of **OCTOBER 1998**

ADAMS & ADAMS
APPLICANTS PATENT ATTORNEYS

The duplicate will be returned to the applicant's address for service as proof of lodging but is not valid unless endorsed with official stamp

OFFICIAL DATE STAMP
1998 -10- 15
REGISTRAR OF PATENTS

A&A P201

ADAMS & ADAMS
PRETORIA

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978
DECLARATION AND POWER OF ATTORNEY
(Section 30 - Regulation 8, 22(i)(c) and 33)

REPUBLIC OF SOUTH AFRICA REVENUE FORM P.3
1.9.98 R 001.00

PATENT APPLICATION NO		
21	01	989427

A&A Ref: V13065

LODGING DATE
22 15 OCTOBER 1998

FULL NAME(S) OF APPLICANT(S)	
71	PROTEIN RESEARCH TRUST

FULL NAME(S) OF INVENTOR(S)	
72	JACOBA ADRIANA DE RONDE DR WILLIAM CRESS

EARLIEST PRIORITY CLAIMED	COUNTRY		NUMBER		DATE	
	33	NIL	31	NIL	32	NIL

NOTE: The country must be indicated by its International Abbreviation - see schedule 4 of the Regulations

TITLE OF INVENTION	
54	TRANSFORMATION PROCESS

* I/We G J H SCHOLTEMEIJER

hereby declare that :-

~~1 I/we am/are the applicant(s) mentioned above;~~

** 2 I/we have been authorized by the applicant(s) to make this declaration and have knowledge of the facts herein stated in the capacity of VICE CHAIRMAN of the applicant(s);

*** 3 the inventor(s) of the abovementioned invention is/are the person(s) named above and the applicant(s) has/have acquired the right to apply by virtue of an assignment from the inventor(s);

4 to the best of my/our knowledge and belief, if a patent is granted on the application, there will be no lawful ground for the revocation of the patent;

**** ~~5 this is a convention application and the earliest application from which priority is claimed as set out above is the first application in a convention country in respect of the invention claimed in any of the claims;~~ and

6 the partners and qualified staff of the firm of **ADAMS & ADAMS**, patent attorneys, are authorised, jointly and severally, with powers of substitution and revocation, to represent the applicant(s) in this application and to be the address for service of the applicant(s) while the application is pending and after a patent has been granted on the application.

SIGNED AT PRETORIA THIS 31ST DAY OF AUGUST 1998

Company Name: PROTEIN RESEARCH TRUST
Full Names: G J H SCHOLTEMEIJER
Capacity: VICE CHAIRMAN

(no legalization necessary)

* In the case of application in the name of a company, partnership or firm, give full names of signatory/signatories, delete paragraph 1, and enter capacity of each signatory in paragraph 2.
** If the applicant is a natural person, delete paragraph 2.
*** If the right to apply is not by virtue of an assignment from the inventor(s), delete "an assignment from the inventor(s)" and give details of acquisition of right.
**** For non convention applications, delete paragraph 5.

A & A. Ref No: V13065

ADAMS & ADAMS
PATENT ATTORNEYS
PRETORIA

FORM P6

REPUBLIC OF SOUTH AFRICA
Patents Act, 1978

PROVISIONAL SPECIFICATION

(Section 30 (1) - Regulation 27)

21	01	OFFICIAL APPLICATION NO

989427

22	LODGING DATE

15 OCTOBER 1998

71	FULL NAME(S) OF APPLICANT(S)

PROTEIN RESEARCH TRUST

72	FULL NAME(S) OF INVENTOR(S)

JACOBA ADRIANA DE RONDE
DR WILLIAM CRESS

54	TITLE OF INVENTION

TRANSFORMATION PROCESS

A&A P2/6

THIS INVENTION relates to a method of transforming plants and to plants transformed by the method.

In one embodiment of the invention, there is provided a method for transforming or genetically engineering plants comprising the steps of :

germinating plant seed for a predetermined period; and

transforming the germinating plant seed by exposing or contacting the germinating plant seed with a suitable wetting agent and an *Agrobacterium* strain to obtain transformed plant seed.

The germinating plant seed may be transformed by the introduction of foreign DNA via the *Agrobacterium* strain. The germinating plant seed may be transformed by exposing or contacting the germinating or germinated plant seed with a culture of *Agrobacterium*, said *Agrobacterium* strain being transformation competent and including a construct comprising a foreign gene, the foreign gene including appropriate regulatory sequences so as to expressed in the cells of a plant which grows from the transformed plant seed.

The *Agrobacterium* strain may be any suitable strain such as *Agrobacterium tumefaciens*, for example, *Agrobacterium tumefaciens* strain LBA4404 deposited at Centraalbureae voor Schimmel-cultures (CBS) in the Netherlands under No. CBS 191.83 on 24 February 1983.

The foreign gene may be any suitable gene such as genes which confer disease resistance and/or drought resistance.

The plant seed may be germinated at a temperature of 22 - 32°C, for example, 29°C, for a period of 2 to 5 days, for example, 4 days.

A culture of *Agrobacterium tumefaciens* and said wetting agent may be mixed together to obtain an *Agrobacterium*/wetting agent suspension. The *Agrobacterium*/wetting agent suspension may comprise 0,01 - 1% of wetting agent, for example, 0,1% wetting agent, and 99,99 - 99% *Agrobacterium tumefaciens*, for example, 99,9 % *Agrobacterium tumefaciens*.

The germinating plant seed may be exposed or contacted with the *Agrobacterium*/wetting agent suspension for a period of 2 - 48 hours, for example, 24 hours, at a temperature of 15 - 35°C, for example, at room or ambient temperature.

The method may include subjecting the germinating seed to vacuum infiltration while the germinating seed is exposed or contacted with the

Agrobacterium/wetting agent suspension. The germinating seed may be subjected to vacuum infiltration for a period of 5 - 40 minutes, for example, 20 minutes, at a pressure of 20 - 100 millitorr, for example, 78 millitorr pressure. The vacuum infiltration may be carried out at a temperature of 15 - 35°C, for example, 25°C.

The *Agrobacterium* strain may include a suitable plasmid to facilitate transformation of the plant seed.

The plasmid may include a vector, such as vector pBI121.

The method may include inducing further growth of the transformed plant seed and selecting for a transformant in the presence of a selecting agent. The *Agrobacterium* strain may include a plasmid comprising both said foreign gene or genes and a selection agent resistance gene, both genes including appropriate regulatory sequences so as to be expressed in the plant arising from the transformed plant seed.

The selection agent may be an antibiotic or antibiotics and the resistance gene may code for antibiotic resistance. The antibiotic may be selected from the group consisting of at least one of kanamycin and rifampicin. The resistance gene may be a GUS-intron gene.

It will be appreciated that any suitable plant seed may be transformed using the method as herein described. The plant seed may be from the family

leguminoseae or any other dicotyledonous plant, for example, soybean seed. If soybean seed is used, the soybean seed may be allowed to germinate until it has a small plumule, easily removable seed coat and cotyledons which are not appressed against each other before the germinating soybean seed is subjected to the transformation step.

The wetting agent may be any suitable wetting agent which facilitates or enhances penetration and transformation of plant seed by the *Agrobacterium* strain. Preferably, the wetting agent is Break-Thru (available from Goldschmidt Chemical Corporation in Hopewell, USA. It is believed that the active component of Break-Thru is polyether polymethyl siloxane copolymer, Break-Thru, being a non-oil wetting agent.

According to another aspect of the invention, there is provided a transformed plant seed produced by the method as herein described.

According to a further aspect of the invention, there is provided a transformed plant produced by the method as herein described.

According to yet another aspect of the invention, there is provided a transformed plant grown or germinated from the transformed plant seed as herein described.

The transformed plant may comprise cells which comprise in their genome at least one preselected foreign gene which produces a foreign cellular product encoded by the foreign gene. The foreign gene may code for at least one of disease resistance or drought resistance.

According to another aspect of the invention, there is provided a plant seed produced by the transformed plant as herein described.

According to a further aspect of the invention, there is provided a plant which is the progeny of a transformed plant as herein described.

The invention will now be described by way of non-limiting example, with reference to the following example of a method of transforming plant seed, in accordance with the invention.

EXAMPLE

Agrobacterium tumefaciens strain LBA4404 containing a GUS-gene (pBI121) was cultured at 27°C in 100 ml Luria-Bertani broth (LB) pH 7.00 supplemented with 150μg/ml rifampicin and 100μg/ml kanamycin until an absorbence of A_{600} = 0.5 was obtained. 0.01 mg/ml Acetosyringone was added to the *Agrobacterium tumefaciens* culture approximately 24 hours before transformation of plant seeds was carried out. The *Agrobacterium tumefaciens* culture was centrifuged at 10000 rpm for 20 minutes at a temperature of 10°C. Flocculation was avoided or

inhibited by dilution of the *Agrobacterium tumefaciens* culture with distilled water to obtain a ratio of *Agrobacterium tumefaciens* : distilled water of 1:4. 0.1% Break-Thru (a wetting agent) (obtainable from Goldschmidt Chemical Corporation was added to the diluted *Agrobacterium tumefaciens* culture.

Soybean seeds were sterilised for 5 minutes in 3.5% (v/v) NaOCl, and then washed in sterile water before being germinated on sterile 0.8% water agar at a temperature of 29°C for a period of 2 to 5 days. The germinating soybean seeds were then sorted and soybean seeds having a small plumule, easily removable seed coat and cotyledons which were not appressed against each other, were selected. The selected germinating or germinated soybean seeds were then contacted with the *Agrobacterium*/ wetting agent suspension and vacuum infiltrated for 20 minutes under a pressure of 78 millitorr. After vacuum infiltration, the germinating soybean seeds were then incubated for a further period of 24 hours in contact with the *Agrobacterium*/wetting agent suspension solution at room or ambient temperature to obtain transformed soybean seeds. The transformed soybean seeds were then planted in a soil mixture comprising soil, sand, vermiculite (5:5:3) and grown in a greenhouse to obtain transformed soybean plants. Percentage success rate of transformation was determined by detecting GUS-gene activity using a fluorometric and a histochemical assay :

When the transformed plants had developed a second set of leaves, a leaf from the main apex was tested for expression of the GUS gene. A fluorometric GUS assay (Jefferson, R.A., Kavanagh, T.A. & Bevan, M.W. 1987.

GUS fusion ß - glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO* J. I 6 (13): 3901-3907 was used for screening of all explants for the expression of glucuronidase enzyme. 100 µl sodium phosphate buffer was pipetted into the wells of a microtiter plate. The assay buffer contained 50 mM $NaPO_4$ (pH7.00), 10mM EDTA, 0.1% (v/v) Triton X-100, 10mM mercapto ethanol and 2 M methyl umbilliferyl glucuronide (Sigma). Small pieces of plant tissue were crushed in this buffer and incubated overnight at a temperature of 37°C in the dark. Reactions were visualised on a long wave length UV light box. A histochemical assay was also performed in testing putative transformants. Plant tissue, which tested positive in the fluorescence assay, was incubated overnight at a temperature of 37°C in the dark in a histochemical staining solution. The staining solution contained 50mM $NaPO_4$ (pH7.00), 0.1% (v/v) Triton X-100, 1.04 mM X-Gluc and 0.5% (v/v) DMSO. The plant tissue was subsequently washed in FAA for 10 minutes, followed with a wash in 50% ethanol. The tissue was dehydrated with 100% ethanol and hydrated slowly up to 100% H_2O.

A positive result was obtained, indicating transformed soybean plants.

To verify the insertion of the GUS gene in the plant genome, molecular analysis, i.e. PCR reaction as well as a Southern blot procedure (Maniatis, T. Fritsch, E.F. & Sambrook, J., 1982. *Molecular Cloning : A Laboratory Manual*. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, H.Y.), were conducted with GUS and NPTII specific primers. PCR and Southern blot observations indicated the

presence of the GUS gene in the soybean genome. Plants tested positive up to the third transformant generation.

The method in accordance with the invention resulted in a transformation success rate of approximately 35% of the soybean seeds. This is a relatively high transformation success rate in that conventional techniques usually only have a transformation success rate of less than 5%. By inserting a foreign gene or genes into a plasmid in *Agrobacterium tumefaciens*, the soybean seed may be transformed with the foreign gene. The foreign gene is then included in the cells of a soybean plant which grows from the transformed soybean seed and may then be inherited by its progeny.

Advantages of the invention are that the method is relatively easy to carry out and relatively inexpensive compared to conventional transformation procedures and techniques. As no tissue culture steps are used in the method according to the invention, it is believed that there will be little or no loss of genetic traits which would usually occur as a result of somatic mutations. The method in accordance with the invention can be used for transforming any suitable plant seed with genes of interest or agricultural usefulness, for example, drought resistant or disease resistant genes. The method in accordance with the invention can also be used for producing transgenic plants of other species where routine tissue culture procedures have not yet been established.

DATED THIS 15TH DAY OF OCTOBER 1998

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